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Simultaneous detection of *S*-adenosylmethionine and *S*-adenosylhomocysteine in mouse and rat tissues by capillary electrophoresis

A capillary electrophoresis method for the determination of *S*-adenosylmethionine (SAM) and *S*-adenosylhomocysteine (SAH) in rat liver and kidney and mouse liver is described. The method can also be used to determine SAM in whole blood. The method provides rapid (approximately 16 min sample to sample) resolution of both compounds in perchloric extracts of tissues. Separation was performed by using an uncoated 50 μm ID capillary with 60 cm total length (50 cm to the detector window). Samples were separated at 22.5 kV and the separation running buffer was 200 mM glycine pH 1.8 (with HCl). The method compares favorably to HPLC methods (r^2 = 0.994 for SAM, r^2 = 0.998 for SAH) and has a mass detection limit of about 10 fmol for both SAM and SAH at a signal-to-noise ratio of 3. The method is linear over ranges of 1–100 μM SAM and 1–250 μM SAH. This method can be used to determine tissue concentrations of SAM and SAH, two metabolites that can provide insight into many biological processes.

Keywords: *S*-Adenosylhomocysteine / *S*-Adenosylmethionine / Capillary electrophoresis / Tissue EL 5311

1 Introduction

S-Adenosylmethionine (SAM) is the primary methyl donor for numerous methyltransferase reactions that target molecules including DNA, RNA, proteins, lipids, polysaccharides, and a variety of small molecules [1, 2]. By donation of its methyl group, SAM is converted into *S*-adenosylhomocysteine (SAH). SAH is an inhibitor of most SAM-dependent methyltransferase reactions [2]. Also, a low SAM/SAH ratio can inhibit SAM-dependent transmethylation reactions [3, 4]. The K_i of the reactions (for SAH) can be in the same order of magnitude of the K_m for SAM. Considering this, the SAM/SAH ratio becomes very important. Thus, all enzymatically mediated transfers of methyl groups from SAM either to proteins or to nucleic acids, phospholipids, *etc*., can be affected, in a competitive fashion, by an increase of intracellular SAH, and/or a reduction of the SAM/SAH ratio [4].

SAM can affect other metabolic steps in methionine metabolism. For example, SAM stimulates the product formation of methionine synthase, inactivates betaine homocysteine methyltransferase, and activates cystathionine synthase [3]. Through activation or suppression of the two mammalian genes that encode for methionine adenosyltransferase, SAM may be important in maintaining the differentiated state of liver [5]. Tissue concentrations of SAM and SAH (and hence of SAM/SAH) are also important for protein methylation and stability. For example, myelin basic protein is methylated at the 107 arginine position [6]. The function of protein methylation is unknown but suggested to be needed for stabilization of tertiary structure of proteins. Impairment of protein methyation can come about by inhibition of the methyltransferase resulting from elevation of SAH or a decrease in the concentration of SAM [6]. SAM is also important in oxidative processes. It protects against mitochondrial injury, which prevents mitochondrial oxidant stress and improves ischemia-induced hepatic energy metabolism [7]. A low SAM/SAH ratio in liver has been correlated with global DNA hypomethylation and an increased risk of cancer [8–10]. Also, decreased hepatic SAM concentrations and hypomethylation of certain oncogenes have correlated with the development of hepatocarcinogenesis, which is completely prevented by exogenous administration of SAM [11].

Thus, determination of tissue SAM and SAH can provide insight into many biological processes. There are many published methods for the determination of SAM and SAH in tissues. Pañak *et al.* [12] published a method for the determination of SAM and SAH by capillary electrophoresis (CE). This method, however, was not developed for biological samples. The method of Pañak *et al.* [12]

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Abbreviations: SAH, *S*-adenosylhomocysteine; **SAM**, *S*-adenosylmethionine

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uses a phosphate buffer as background electrolyte. Upon testing a wide range of concentrations and pH of phosphate buffers, we could not resolve SAM and SAH in biological tissue such as liver. Most methods for the determination of SAM and SAH in tissues are HPLC-based and use UV detection [13–17]. Several use fluorescent or electrochemical detection [18, 19]. Precipitation of tissue protein is usually done by addition of perchloric acid (PA) or trichloroacetic acid (TCA). Supernatant from PA-based methods are filtered and then injected directly onto the column [13, 15, 16]. Most procedures that use TCA for protein precipitation extract the TCA with ether prior to injection onto the column. The CE method presented here is rapid (10 min run time plus approximately 6 min between runs), requires very little sample, has the ease of sample preparation, and has the sensitivity and precision needed to determine SAM and SAH in various tissues including kidney, liver and blood (SAM only).

2 Materials and methods*

2.1 Instrumentation and separation conditions

Analysis was performed with a Beckman P/ACE System MDQ Capillary Electrophoresis (Fullerton, CA, USA) equipped with a photodiode array detector; wavelengths of 257 and 205 nm were monitored. The polyimide-coated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA; 50 μm ID × 60 cm total length, 50 cm to the detector window) was maintained at a constant temperature of 25°C. Sample storage was at 4°C. Samples were separated at 22.5 kV and the separation running buffer was 200 mM glycine pH 1.8 (with HCl). Timed program events are shown in Table 1. Sample or standard injection was by pressure and ranged from 0.5 to 3 psi and 10–60 s (see text).

Table 1. Timed program event

Rinse	H_2O	20 psi 1 min
Rinse	0.1 N NaOH	20 psi 2 min
Rinse	H_2O	20 psi 1 min
Rinse	Run buffer	20 psi 1 min
Inject		(by pressure, variable[a)])
Separate	22.5 kV	10 min, 0.17 min ramp

a) See text

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2.2 Enzymatic synthesis of *S*-adenosylmethionine

SAM was enzymatically synthesized by a modified method of Cantoni [20]. Reduced glutathione was not used in the reaction and KCl was added (0.3 M in the final reaction volume). Rat liver methionine adenosyltransferase was purified to the ammonium sulfate precipitate I step by the method of Cantoni and Durell [21]. After 5 h of incubation at 37°C the reaction was terminated with an equal volume of 10% TCA. Following centrifugation the supernatant was extracted three times with an equal volume of ether to remove most of the TCA. The ether-extracted supernatant was applied to a Cellex-P column and SAM was eluted according to Eloranta *et al.* [22]. The fraction containing SAM was evaporated to dryness [23] and brought up in a minimal amount of 50 mM HCl. Because the enzyme preparation used in the synthesis of SAM is not pure and some SAM-dependent methyltransferases are present, a small amount of SAH is formed during the reaction. This fraction was also collected from the Cellex-P chromatography [22].

2.3 Tissue preparation

Mouse liver, rat liver and kidney were excised and homogenized in 0.4 N $HClO_4$ (1 g tissue to 5.0 mL $HClO_4$) by using a tissuemizer (Tekmar, Cincinnati, OH, USA) or a glass homogenizing vessel with a ground glass pestle. Whole blood from rats and mice was deproteinized with an equal volume of 0.667 N perchloric acid. After centrifugation, supernatants were stored at −70°C until analysis. Some samples were divided into two aliquots, one for analysis by HPLC and the other for analysis by CE. Perchloric acid extracts of tissue and blood samples were filtered by using 0.2 or 0.45 μm syringe filters (CE and HPLC) and diluted 50:50 v:v with H_2O prior to analysis (CE only).

2.4 HPLC

HPLC analysis of SAM and SAH was done by the method of Wagner *et al.* [15] on a Shimadzu LC-10 HPLC (Columbia, MD, USA) equipped with a 250 × 4.6 mm Ultrasphere 5 μ C18 IP column (Phenomenex, Torrance, CA, USA). SAM and SAH were detected by absorbance at 254 mn. Tissue samples were prepared as noted above. A gradient elution system was used; mobile phases consisted of sodium phosphate/acetonitrile/octane sulfonic acid.

2.5 Standards

Stock solutions of SAM and SAH were prepared from synthesized SAM or commercial SAH (Sigma Chemical, St. Louis, MO, USA). The amount of SAM or SAH in the

stock solutions was determined by using the molar extinction coefficient of 15 000 L/mol·cm at 257 nm for both substances [22]. Standards (1–500 μM) were prepared in 0.167 N $HClO_4$ to reflect the approximate perchloric concentration in the 50:50 H_2O-diluted tissue samples.

2.6 Precision and statistical analysis

To determine intra-assay precision, 7 replicates of the same sample were analyzed in a single run (Table 2). Interassay precision was determined by analysis of the same sample used for intra-assay determination by analysis on 6 different days over a period of 32 days (Table 2). Statistical differences between means were calculated by using the Student's *t*-test; regressions and correlations were done with Quattro Pro 8 (Corel Ottawa, Ontario, Canada) or Deltagraph 4 (SPSS Chicago, IL, USA).

Table 2. Precision of assay: migration time (MT) and concentration of SAM and SAH in replicates of rat liver

	Intraassay (n = 7)		Interassay (n = 6)	
	SAM	SAH	SAM	SAH
MT, min	7.27 ± 0.01	9.11 ± 0.02	7.29 ± 0.02	9.15 ± 0.02
CV (%)	0.15	0.18	0.21	0.22
Concentration (nmol/g)	67.8 ± 1.21	14.0 ± 0.63	66.5 ± 2.42	14.8 ± 0.90
CV (%)	1.78	4.49	3.64	6.08

Values are means ± SD.
Analytical conditions: 200 mM glycine buffer, pH 1.8 (with HCl); voltage, 22.5 kV; injection, 0.5 psi 60 s; absorbance at 257 nm

3 Results and discussion

Commercial preparations of SAM show a double peak in the electropherogram (Fig. 1); this double peak was not seen in enzymatically synthesized SAM (Fig. 1). The double peak seen in commercial preparations of SAM was also reported by Pañak *et al.* [12] who suggested that the minor peak was an unnatural diastereomer. Because of the presence of the second peak in commercial preparations of SAM, enzymatically synthesized SAM was used as a standard throughout this study. Enzymatically synthesized SAM shows a very small peak migrating at the same time as the secondary peak seen in commercial preparations of SAM (Fig. 1, insert). This may be an unnatural diastereomer as suggested above. Commercially prepared SAH was compared by HPLC, CE, and by comparison of UV/Vis spectrum (190–300 nm) to enzymatically synthesized SAH. Because the commercially prepared and enzymatically synthesized SAM seemed to be identical, commercially prepared SAH was used.

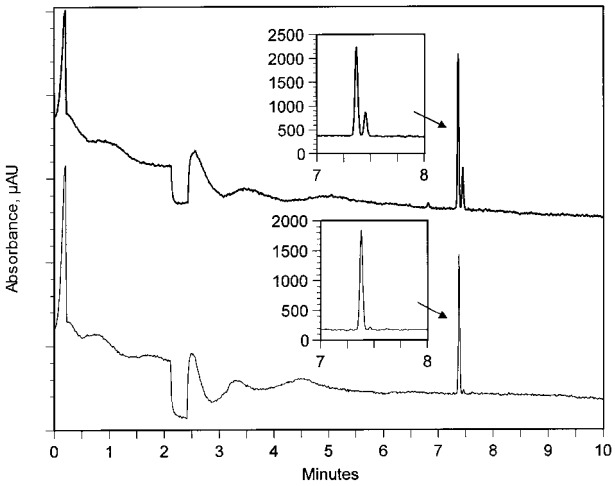

Figure 1. Top: electropherogram of commercially prepared SAM (~ 25 μM); bottom: electropherogram of enzymatically synthesized SAM (~ 10 μM). Inserts: expanded views of double peak region associated with commercial SAM. Analytical conditions: 200 mM glycine buffer, pH 1.8 (with HCl); voltage, 22.5 kV; injection, 0.5 psi 30 s; absorbance at 257 nm.

Standard curves of 25–250 μM SAM were run. SAM shows linearity to about 100 μM ($y = -0.913x^2 + 841x$, $r^2 = 0.916$; y = peak area, x = SAM concentration), which corresponds to approximately 8 pmol mass limit (under injection conditions of 0.5 psi for 60 s). A standard curve for SAH (25–500 μM) shows linearity to about 250 μM ($y = -0.254x^2 + 937x + 656$, $r^2 = 0.931$; y = peak area, x = SAH concentration) corresponding to about 19 pmol mass limit. To better reflect tissue concentrations, standard curves based on 1, 2.5, 5, 7.5, 10, and 25 μM SAM or SAH were run. These standard curves show excellent fits (SAM, $y = 730x - 175$; SAH, $y = 979x - 57.2$; $r^2 = 0.999$, for both). Typically, standard concentrations of 10 μM or less are used when tissue samples are run. An approximation of a mass detection limit for both SAM and SAH was calculated to be about 10 fmol at a signal-to-noise ratio of 3; this mass detection limit was similar to that seen by Pañak *et al.* [12]. The intra-assay and interassay precision for migration times and concentrations of SAM and SAH in replicates of rat liver are summarized in Table 2. These data suggest that this CE method is consistent and reliable.

Rat and mouse liver samples were spiked with SAM and SAH to verify peaks. Figure 2 shows electropherograms of rat liver (Fig. 2A) and mouse liver (Fig. 2B) sam-

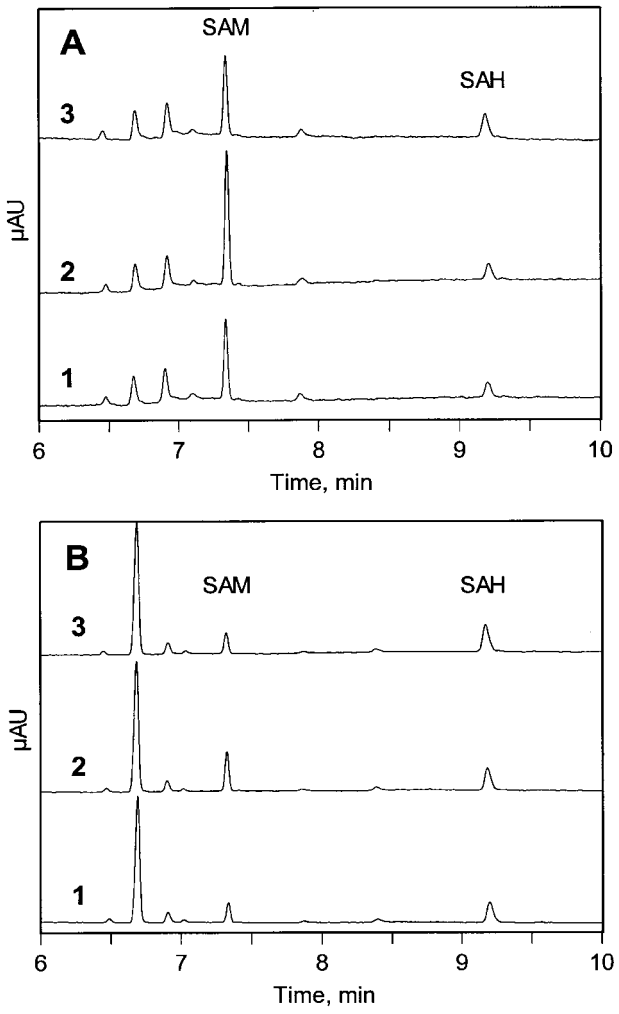

Figure 2. (A) 1: electropherogram of rat liver extract; 2: rat liver extract spiked with SAM; 3: rat liver extract spiked with SAH. (B) 1: Electropherogram of mouse liver extract; 2: mouse liver extract spiked with SAM; 3: mouse liver extract spiked with SAH. Analytical conditions as in Fig. 1.

ples monitored at 257 nm and run separately or spiked with either SAM or SAH. Data collected from 205 nm were also used to verify the peaks (data not shown). Under these conditions, SAM and SAH migrate as single peaks.

The effect of various injection pressures on detection of SAM and SAH in rat and mouse liver at 257 and 205 nm was determined. At an injection of 60 s, pressures of 0.5, 1, 2 and 3 psi result in approximately 59, 118, 237, or 355 nL injection volumes, respectively, representing 6, 12, 24, or 36% sample plug to window. The current varied from 220–240 μA with a 0.5 psi injection, 210–240 μA with a 1 psi injection, 160–250 μA with a 2 s injection, and 135–280 μA with a 3 s injection. The SAM peak as

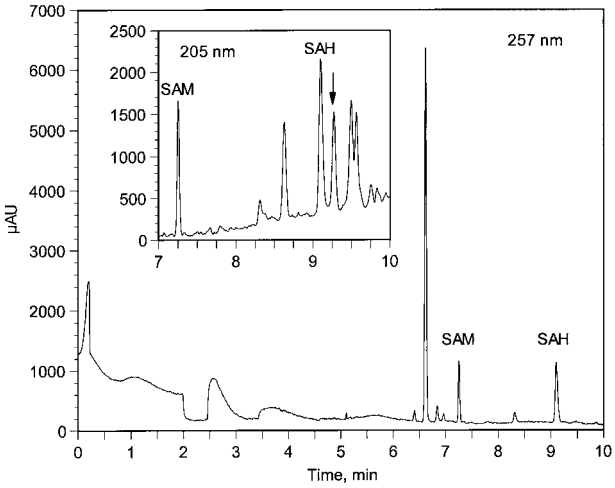

Figure 3. Electropherogram of mouse liver monitored at 257 nm. Insert: corresponding electropherogram recorded at 205 nm. Arrow denotes peak seen at 205 nm, but not 257 nm; this peak can interfere with resolution of SAH under conditions of excess sample load (see text). Analytical conditions as in Fig. 1.

detected at 257 or 205 nm was sharp and well resolved regardless of pressure. As monitored at 257 nm, SAH seemed to be resolved at 0.5, 1 and 2 psi. However, detection at 205 nm revealed a peak migrating just after SAH (designated by arrow in Fig. 3, insert). This peak and SAH tend to comigrate at pressures of 2 and 3 psi. Therefore, an injection time and pressure corresponding to a maximum of ~12% sample plug to window was used. Electropherograms of SAM and SAH of a mouse liver sample monitored at 257 and 205 nm (0.5 psi 60 s injection) are shown in Figure 3. Thus, typical runs were done with 60 s injections at 0.5 psi which resulted in well-defined and resolved SAM and SAH peaks.

Figure 4 shows the correlation between HPLC and CE methods for mouse liver SAM and SAH. The data show very good correlation between CE and HPLC for both

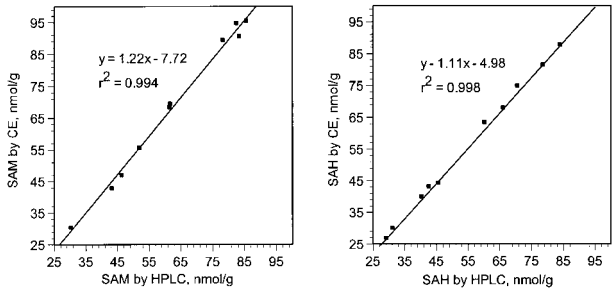

Figure 4. Left panel: correlation of SAM in mouse liver determined by CE and HPLC. Right panel: correlation of SAH in mouse liver determined by CE and HPLC. CE analytical conditions as in Fig. 1.

SAM (r^2 = 0.994) and SAH (r^2 = 0.998). Although the HPLC and CE methods correlate very strongly for SAM, the slope is different from 1. Also, a paired *t*-test indicates that the mean for the CE method differed from the mean for the HPLC method (68.3 *vs*. 62.5, $p < 0.003$). Thus, even though the methods correlate strongly, they should not be used interchangeably. Comparisons of HPLC to CE were also done on several tissues from a limited number of rats. These data are shown in Table 3 and support the correlation between HPLC and CE methods.

Table 3. Comparison of SAM and SAH from rat liver and kidney as determined by CE and HPLC

	SAM (nmol/g)		SAH (nmol/g)	
	HPLC	CE	HPLC	CE
Liver	77.8 ± 5.0	76.6 ± 7.2	16.8 ± 3.2	16.2 ± 4.5
Kidney	34.9 ± 2.8	34.4 ± 3.3	10.6 ± 2.0	8.8 ± 2.8

Samples prepared (see text) and divided into two aliquots, one for HPLC and one for CE. Values are means ± SD, *n* = 3. Analytical conditions were as in Table 2.

An electropherogram of SAM in mouse whole blood is shown in Fig. 5. Because of the low concentrations of SAM and SAH in whole blood, samples were injected for 60 s at 1 psi (up to 2 psi 60 s injection still produced an acceptable SAM peak but no detectable SAH peak). The sample, spiked with SAM and SAH is shown in the top panel of Fig. 5. Data generated at 205 nm were also used to confirm the presence of SAM and nondetection of SAH in mouse blood by CE (data not shown). Thus, SAM can be detected by using this CE method but the concentration of SAH in blood seems to be too low to be detected under these conditions. The HPLC-method of Wise *et al.* [17] was used to measure SAM in whole blood and red blood cells. SAM was not detected in plasma by this method. Sample preparation was with TCA followed by washing with ether. Sample preparation of whole blood for determination of SAM by CE is very easy. By CE, the concentration of SAM in whole blood from one mouse sample was 6.06 μM; the concentration from several samples of rat blood ranged from 2.2 to 3.5 μM. Literature values of SAM in blood of mice range from 8.08–8.56 μM [17] and for rats from 4.93–7.91 μM [17]. Values of SAM determined by CE seem to be somewhat lower than the few reported literature values; however, diet and strain of animal can impact the amount of SAM found in blood.

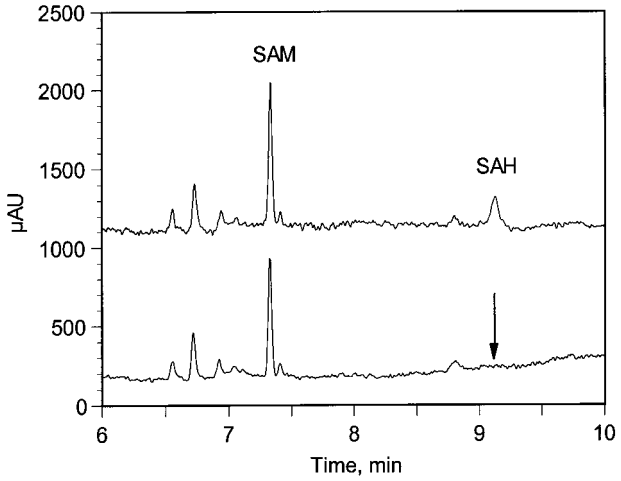

Figure 5. Bottom: electropherogram of SAM (corresponding to ~6 μM) in mouse whole blood (no SAH detected, arrow); top: mouse blood sample spiked with SAM and SAH (SAM and SAH concentrations correspond to ~8 μM and 1 μM, respectively). Analytical conditions as in Fig. 1.

This CE method was developed to determine SAM and SAH in tissues. The method can be used for liver and kidney. In addition, whole blood SAM but not SAH can be determined with this method. It is expected that SAM and SAH from other tissues could be determined easily. For example, SAM and SAH in a rat colon sample was measured and the results compared favorably those obtained by HPLC. The CE method is fast, sensitive and reliable. It requires no column equilibration or regeneration as needed in HPLC-based methods, uses no organic solvents, and requires only simple sample preparation. Thus, this method can be used to determine tissue concentrations of SAM and SAH, two metabolites that can provide insight into many biological processes.

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